

PATENT APPLICATION

**INHIBITORS OF EPOXIDE HYDROLASES FOR THE TREATMENT
OF HYPERTENSION**

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INHIBITORS OF EPOXIDE HYDROLASES FOR THE TREATMENT OF HYPERTENSION

CROSS-REFERENCES TO RELATED APPLICATIONS

5 [0001] This application is a continuation of U.S. Application No. 10/328,495, filed December 23, 2002, which is a continuation of U.S. Application No. 09/721,261, now U.S. Patent No. 6,531,506, which is a continuation in part of U.S. Application No 09/252,148, filed February 18, 1999, now U.S. Patent No. 6,150,415. The disclosures of all of these
10 applications are incorporated herein by reference.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0002] This invention was made with Government support under Grant Nos. HL53994,
15 ES02710, and ES04699 awarded by the National Institutes of Health.

BACKGROUND OF THE INVENTION

Field of the Invention

[0003] The present invention generally relates to methods of treating hypertension using
20 inhibitors of epoxide hydrolases. Preferred inhibitors include compounds, such as ureas, amides, and carbamates that can interact with the enzyme catalytic site and mimic transient intermediates. Other useful inhibitors include glycodiols and chalcone oxides which can interact with the enzyme as irreversible inhibitors.

Background of the Invention

[0004] Hypertension is the most common risk factor for cardiovascular disease, the leading
cause of death in many developed countries. Essential hypertension, the most common form of hypertension, is usually defined as high blood pressure in which secondary causes such as renovascular disease, renal failure, pheochromocytoma, aldosteronism, or other causes are
25 not present (for a discussion of the definition and etiology of essential hypertension *see*, Carretero and Oparil *Circulation* 101:329-335 (2000) and Carretero, O.A. and S. Oparil.. *Circulation* 101:446-453 (2000))

[0005] A combination of genetic and environmental factors contribute to the development of hypertension and its successful treatment is limited by a relatively small number of

therapeutic targets for blood pressure regulation. Renal cytochrome P450 (CYP) eicosanoids have potent effects on vascular tone and tubular ion and water transport and have been implicated in the control of blood pressure (Makita *et al. FASEB J* 10:1456-1463 (1996)).

The major products of CYP-catalyzed arachidonic acid metabolism are regio- and

5 stereoisomeric epoxyeicosatrienoic acids (EETs) and 20-hydroxyeicosatetraenoic acid (20-HETE). 20-HETE produces potent vasoconstriction by inhibition of the opening of a large-conductance, calcium-activated potassium channel leading to arteriole vascular smooth muscle depolarization (Zou *et al. Am J. Physiol.* 270:R228-237 (1996)). In contrast, the EETs have vasodilatory properties associated with an increased open-state probability of a
10 calcium-activated potassium channel and hyperpolarization of the vascular smooth muscle and are recognized as putative endothelial derived hyperpolarizing factors (Campbell *et al. Cir. Res.* 78:415-423 (1996)). Hydrolysis of the EETs to the corresponding dihydroxyeicosatrienoic acids (DHETs) is catalyzed largely by soluble epoxide hydrolase (sEH) (Zeldin *et al. J. Biol. Chem.* 268:6402-64-07 (1993)).

15 [0006] Recent studies have indicated that renal CYP-mediated 20-HETE and EET formation are altered in genetic rat models of hypertension and that modulation of these enzyme activities is associated with corresponding changes in blood pressure (Omata *et al. Am J Physiol* 262:F8-16 (1992); Makita *et al. J Clin Invest* 94:2414-2420 (1994); Kroetz *et al. Mol Pharmacol* 52:362-372 (1997); Su, P. et al., *Am J Physiol* 275, R426-438 (1998)).

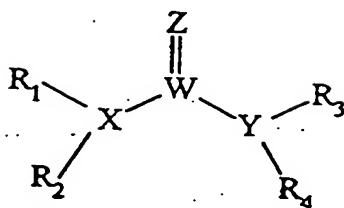
20 Modulation of the CYP pathways of arachidonic acid metabolism as a means to regulate eicosanoid levels is limited by multiple isoforms contributing to a single reaction and the general lack of selectivity of most characterized inhibitors and inducers. Similarly, modulating EET levels by regulation of their hydrolysis to the less active diols has not been considered in light of concerns that EETs are involved in many physiological processes.

25 (Campbell, *Trends Pharmacol Sci* 21:125-7 (2000)).

SUMMARY OF THE INVENTION

[0007] The present invention provides methods of treating hypertension by administering to a patient a therapeutically effective amount of an inhibitor of epoxide hydrolase. A
30 preferred class of compounds for practice in accordance with the invention has the structure shown by Formula 1.

FORMULA 1



wherein Z is oxygen or sulfur, W is carbon phosphorous or sulfur, X and Y is each

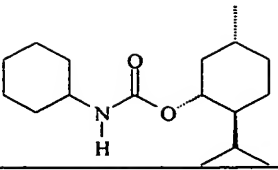
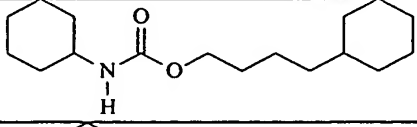
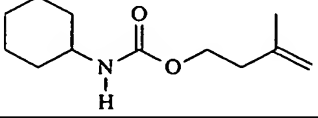
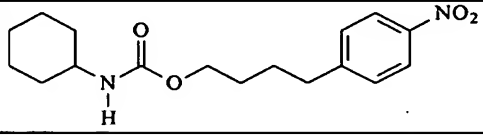
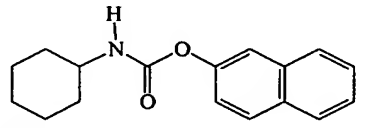
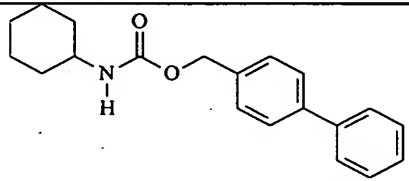
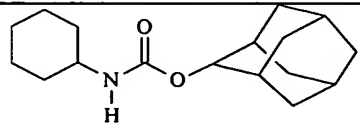
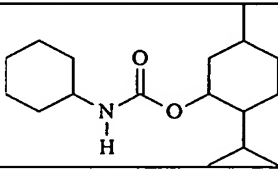
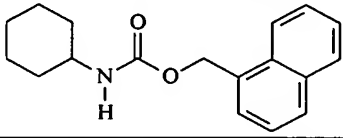
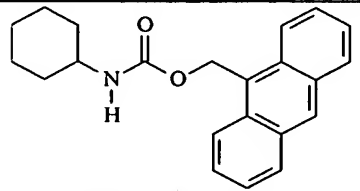
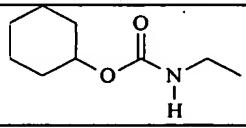
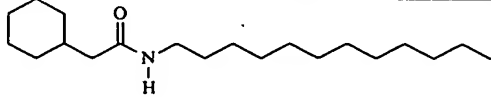
- 5 independently nitrogen, oxygen, or sulfur, and X can further be carbon, at least one of R₁-R₄ is hydrogen, R₂ is hydrogen when X is nitrogen but is not present when X is sulfur or oxygen, R₄ is hydrogen when Y is nitrogen but is not present when Y is sulfur or oxygen, R₁ and R₃ are each independently a substituted or unsubstituted alkyl, haloalkyl, cycloalkyl, aryl, acyl, or heterocyclic.

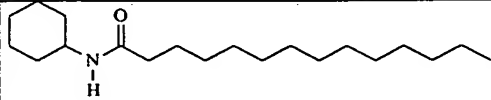
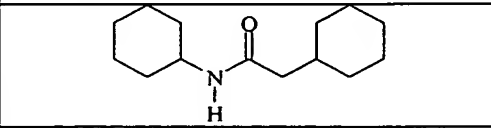
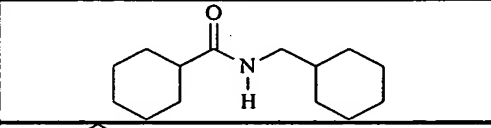
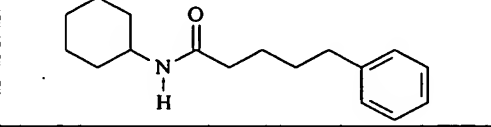
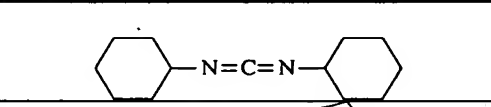
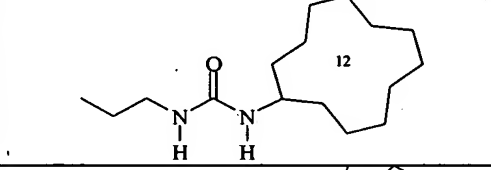
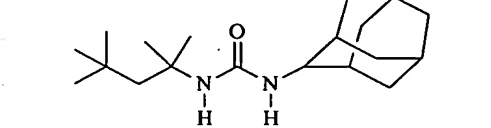
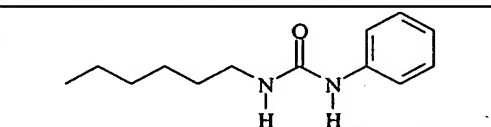
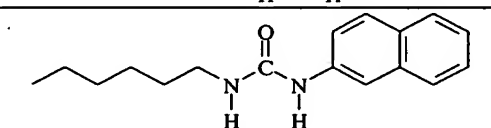
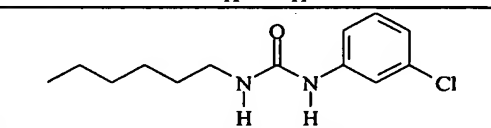
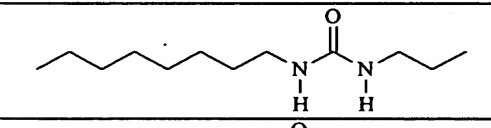
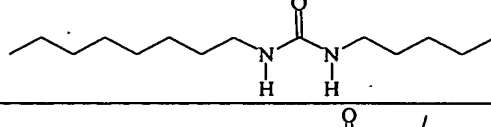
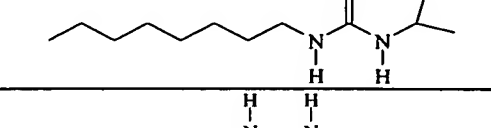
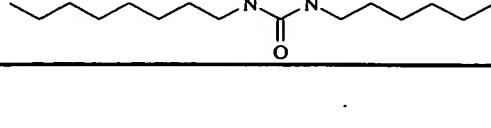
- 10 **[0008]** Preferred compounds of the invention have an IC₅₀ (inhibition potency or, by definition, the concentration of inhibitor which reduces enzyme activity by 50%) of less than about 500 μM. Exemplary compounds of the invention are listed in Table 1. The Table shows inhibition of recombinant mouse sEH (MsEH) and Human sEH (HsEH). The enzyme concentrations were 0.13 and 0.26 micromolar respectively

15 TABLE 1

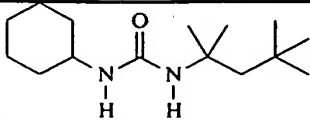
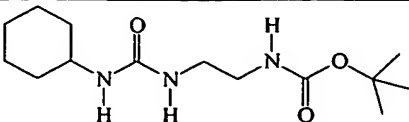
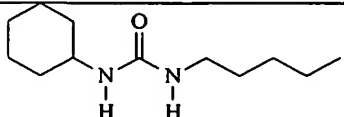
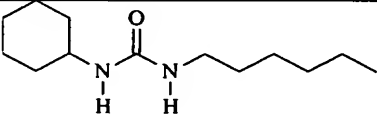
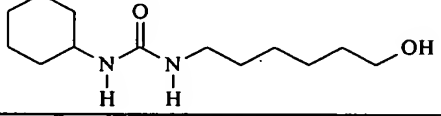
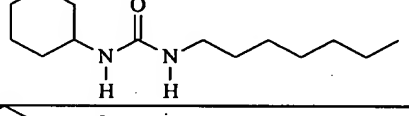
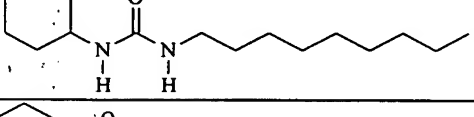
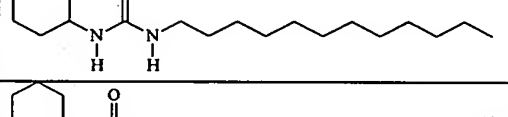
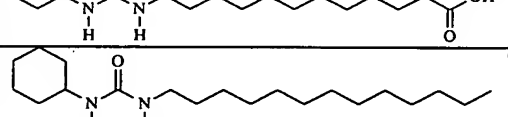
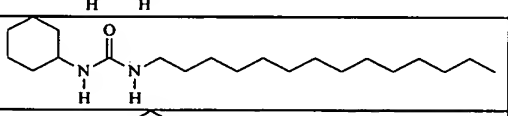
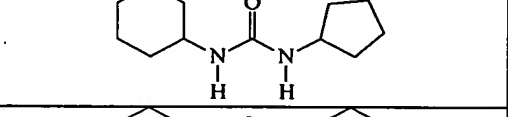
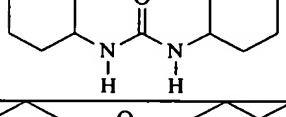
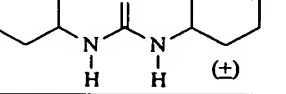

Inhibition of MsEH (0.13 μM) and HsEH (0.26 μM)

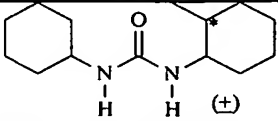
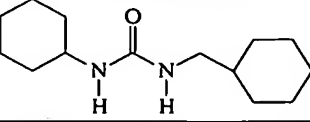
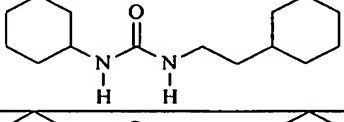
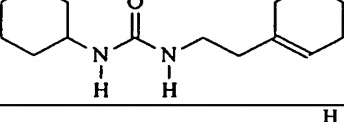
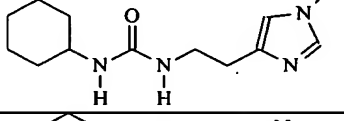
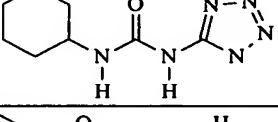
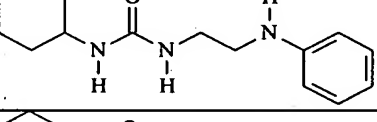
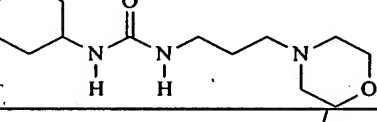
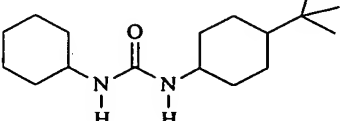
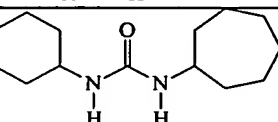
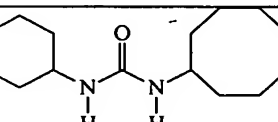
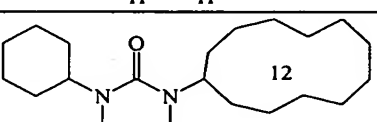
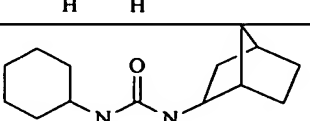
Structure inhibitors	nb	Mouse sEH IC ₅₀ (μM)*	Human sEH IC ₅₀ (μM)*
	72	0.11 ± 0.01	0.48 ± 0.01
	248	0.33 ± 0.05	2.9 ± 0.6
	42	0.06 ± 0.01	0.13 ± 0.01
	224	0.99 ± 0.02	0.32 ± 0.08

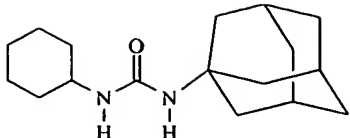
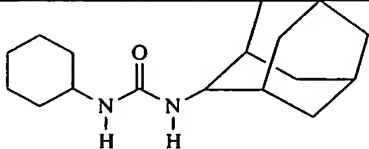
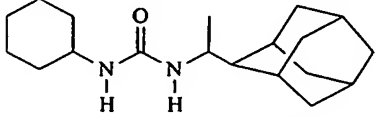
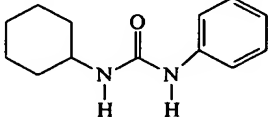
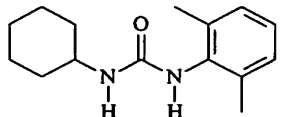
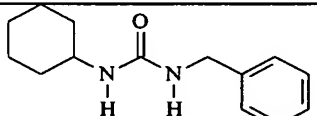
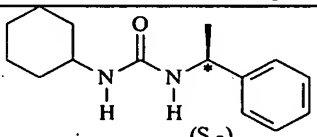
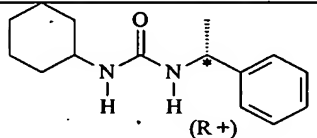
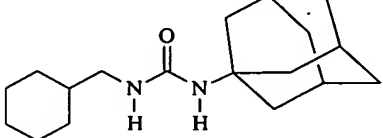
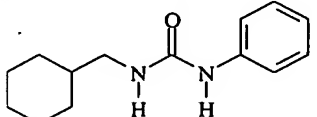
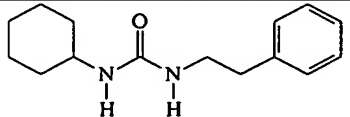
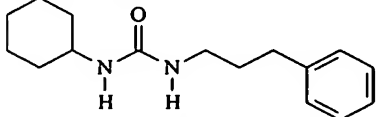
	225	0.84 ± 0.10	1.05 ± 0.03
	276	1.1 ± 0.1	0.34 ± 0.02
	277	0.12 ± 0.01	0.22 ± 0.02
	460	0.10 ± 0.02	0.18 ± 0.01
	110	0.21 ± 0.01	0.35 ± 0.01
	259	0.45 ± 0.09	0.10 ± 0.02
	260	0.06 ± 0.01	0.10 ± 0.01
	261	0.08 ± 0.01	0.12 ± 0.01
	262	0.05 ± 0.01	0.10 ± 0.02
	263	3.0 ± 0.3	0.33 ± 0.06
	255	0.48 ± 0.07	2.88 ± 0.04
	514	0.27 ± 0.01	

	515	0.19 ± 0.05	
	187	0.05 ± 0.01	0.42 ± 0.03
	374	0.25 ± 0.01	2.03 ± 0.07
	381	0.06 ± 0.01	0.68 ± 0.03
		0.25 ± 0.02	0.47 ± 0.01
	189	0.80 ± 0.03	1.0 ± 0.2
	375	0.18 ± 0.01	0.11 ± 0.01
	157	0.85 ± 0.01	1.43 ± 0.03
	143	1.0 ± 0.1	0.57 ± 0.01
	178	0.31 ± 0.01	0.25 ± 0.01
	380	0.73 ± 0.03	0.68 ± 0.03
	125	0.09 ± 0.01	0.72 ± 0.02
	183	1.06 ± 0.07	5.9 ± 0.3
	175		0.24 ± 0.01

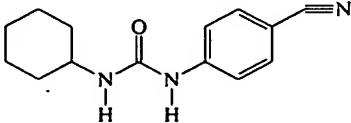
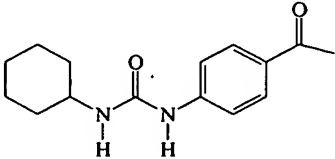
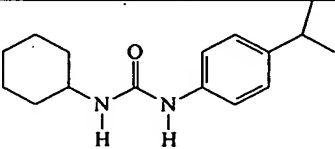
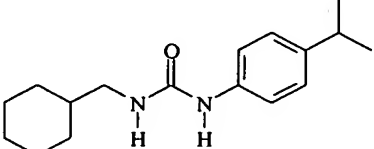
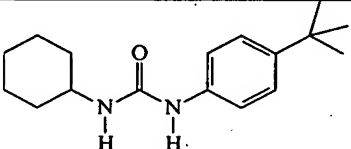
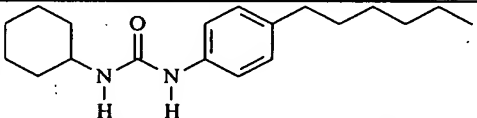
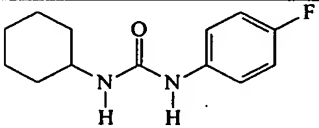
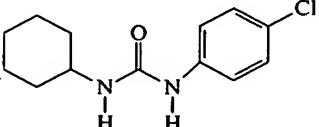
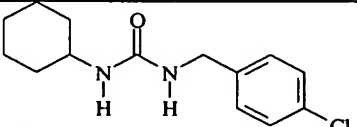
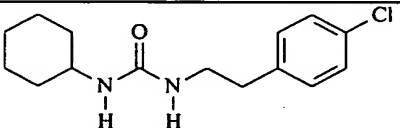
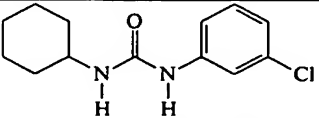
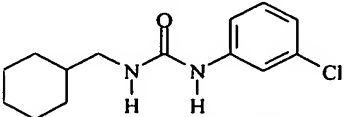
	181	0.52 ± 0.02	1.71 ± 0.23
	168	0.06 ± 0.01	0.12 ± 0.01
	151	2.29 ± 0.03	0.58 ± 0.01
	170	0.12 ± 0.01	0.18 ± 0.01
	429	0.38 ± 0.04	1.7 ± 0.4
	153	0.06 ± 0.01	0.10 ± 0.01
	148	0.21 ± 0.01	0.61 ± 0.02
	172	0.12 ± 0.01	0.30 ± 0.01
	556	0.20 ± 0.02	0.74 ± 0.07
	478	0.05 ± 0.01	0.26 ± 0.02
	562	0.5 ± 0.1	15 ± 3
	531	0.14 ± 0.02	0.64 ± 0.03
	504	0.8 ± 0.1	23 ± 4
	479	0.60 ± 0.06	5.0 ± 0.1
	103	0.12 ± 0.01	2.2 ± 0.1

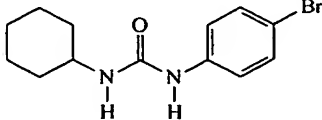
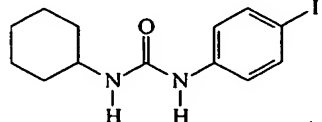
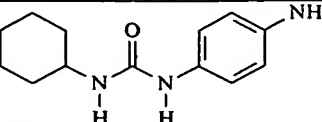
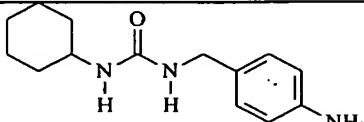
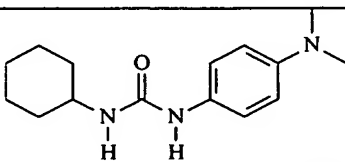
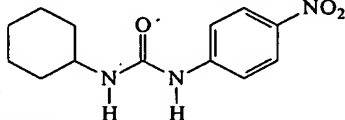
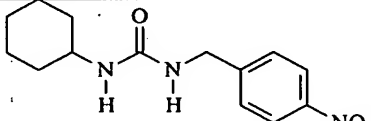
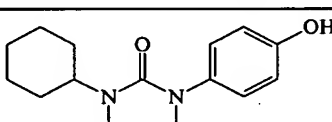
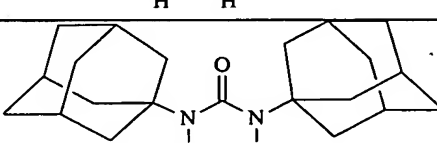
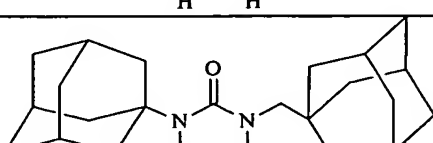
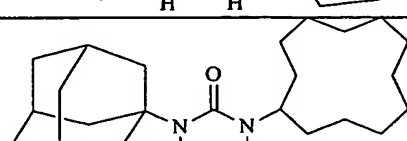
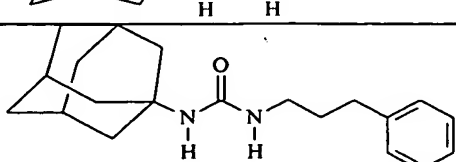
	347	0.07 ± 0.01	3.10 ± 0.07
	124	0.05 ± 0.01	0.14 ± 0.01
	509	0.06 ± 0.01	0.92 ± 0.08
	286	0.11 ± 0.03	0.07 ± 0.02
	344	0.05 ± 0.01	2.50 ± 0.08
	508	0.05 ± 0.01	0.10 ± 0.01
	473	0.05 ± 0.01	0.10 ± 0.01
	297	0.05 ± 0.01	0.10 ± 0.01
	425	0.05 ± 0.01	0.10 ± 0.01
	354	0.05 ± 0.01	0.10 ± 0.01
	477	0.11 ± 0.01	0.24 ± 0.01
	23	0.10 ± 0.01	1.69 ± 0.05
		0.09 ± 0.01	0.16 ± 0.01
	538	0.05 ± 0.01	0.10 ± 0.01

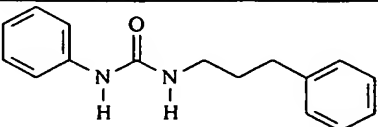
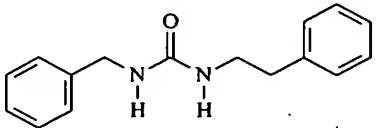
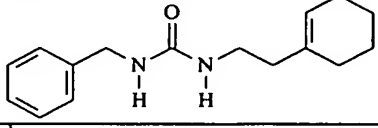
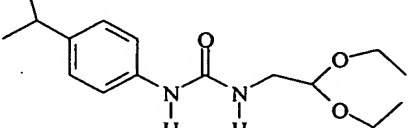
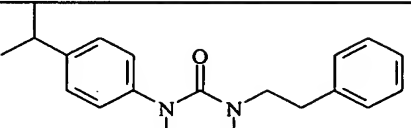
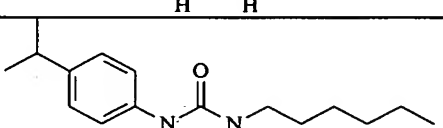
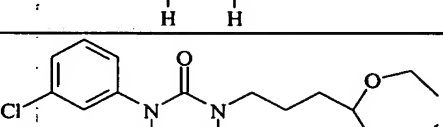
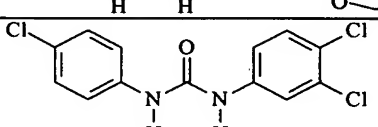
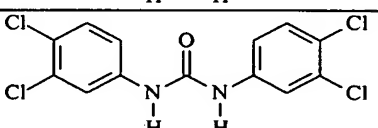
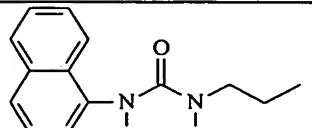
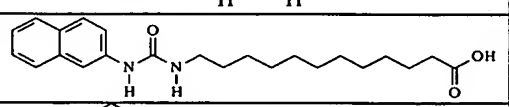
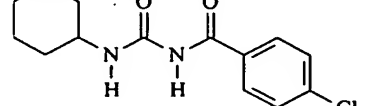
	551	0.05 ± 0.01	0.10 ± 0.01
	57	0.06 ± 0.01	0.16 ± 0.01
	360	0.05 ± 0.01	0.10 ± 0.01
	359	0.05 ± 0.01	0.10 ± 0.01
	461	0.42 ± 0.01	0.55 ± 0.02
	533	0.05 ± 0.1	0.10 ± 0.01
	463	0.90 ± 0.07	8.3 ± 0.4
	377	0.7 ± 0.1	17.8 ± 0.7
	428	0.05 ± 0.1	0.10 ± 0.01
	22	0.05 ± 0.1	0.10 ± 0.01
	58	0.05 ± 0.01	0.09 ± 0.01
	119	0.05 ± 0.01	0.10 ± 0.01
	543	0.05 ± 0.01	0.10 ± 0.01

	192	0.05 ± 0.01	0.10 ± 0.01
	427	0.05 ± 0.01	0.10 ± 0.01
	358	0.05 ± 0.01	0.18 ± 0.04
	21	0.76 ± 0.02	1.39 ± 0.02
	435	0.05 ± 0.01	0.18 ± 0.01
	270	0.05 ± 0.01	1.9 ± 0.1
 (S-)	544	0.05 ± 0.01	0.10 ± 0.01
 (R+)	545	0.05 ± 0.01	3.7 ± 0.3
	437	0.05 ± 0.01	0.10 ± 0.01
	176	0.06 ± 0.01	0.53 ± 0.03
	36	0.06 ± 0.01	0.16 ± 0.02
	104	0.04 ± 0.01	0.29 ± 0.01

	105	0.05 ± 0.01	0.58 ± 0.03
	100	0.07 ± 0.01	0.15 ± 0.01
	188	0.73 ± 0.08	2.50 ± 0.03
	434	0.05 ± 0.01	0.10 ± 0.01
	59	0.85 ± 0.02	0.48 ± 0.01
	559	0.08 ± 0.01	0.14 ± 0.01
	169	0.06 ± 0.01	0.13 ± 0.01
	140	0.05 ± 0.01	0.10 ± 0.01
	108	0.13 ± 0.01	0.17 ± 0.01
	67	0.71 ± 0.04	0.23 ± 0.01
	384	0.05 ± 0.01	1.0 ± 0.2
	343	0.05 ± 0.01	0.10 ± 0.01

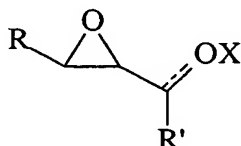
	118	0.06 ± 0.01	0.10 ± 0.01
	126	0.06 ± 0.01	0.27 ± 0.02
	66	0.09 ± 0.01	0.07 ± 0.01
	180	0.06 ± 0.01	0.10 ± 0.01
	75	0.06 ± 0.01	0.23 ± 0.01
	501	0.05 ± 0.01	0.16 ± 0.01
	60	0.78 ± 0.02	0.43 ± 0.02
	20	0.19 ± 0.02	0.40 ± 0.05
	193	0.05 ± 0.01	0.19 ± 0.01
	361	0.07 ± 0.02	0.20 ± 0.02
	44	0.07 ± 0.01	0.19 ± 0.01
	179	0.06 ± 0.01	0.10 ± 0.01

	65	0.17 ± 0.01	0.11 ± 0.01
	53	0.07 ± 0.01	0.12 ± 0.01
	385	0.17 ± 0.02	2.2 ± 0.1
	379	0.05 ± 0.01	1.5 ± 0.1
	362	0.05 ± 0.01	1.5 ± 0.1
	38	0.17 ± 0.01	0.36 ± 0.02
	341	2.3 ± 0.3	4.3 ± 0.4
	128	0.79 ± 0.08	11.1 ± 0.8
	411	0.05 ± 0.01	0.10 ± 0.01
	412	0.05 ± 0.01	0.10 ± 0.01
	413	0.05 ± 0.01	0.10 ± 0.01
	438	0.05 ± 0.01	0.10 ± 0.01

	430	0.21 ± 0.02	0.55 ± 0.03
	470	0.59 ± 0.08	7.6 ± 0.1
	471	0.25 ± 0.03	2.2 ± 0.1
	159	0.59 ± 0.03	3.40 ± 0.04
	156	0.20 ± 0.01	0.48 ± 0.01
	287	0.09 ± 0.01	0.10 ± 0.01
	167	0.39 ± 0.02	3.77 ± 0.03
		0.36 ± 0.02	0.12 ± 0.01
	299	0.7 ± 0.1	0.26 ± 0.02
	253	0.10 ± 0.02	0.28 ± 0.01
	283	0.7 ± 0.2	1.12 ± 0.03
	257	0.05 ± 0.01	0.10 ± 0.04

[0009] A second preferred class of compounds for practice in accordance with the invention has the structure shown by Formula 2,

FORMULA 2

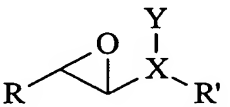


wherein R is alkyl or aryl, the compound is *trans*- across the epoxide ring, OX is a carbonyl (=O) or hydroxy group (OH) and R' is a H, alkyl or aryl group. The preparation of these compounds is described in US Patent No. 5,955,496 and in WO98/06261.

10 [0010] Exemplary compounds are shown in Table 2, below.

TABLE 2

Inhibition of MsEH (0.13 μ M) and HsEH (0.26 μ M)

				Mouse sEH IC ₅₀ (μ M)*	Human sEH IC ₅₀ (μ M)*
R	R'	X-Y	Abs. Conf.		
C ₆ H ₅	C ₆ H ₅	C=O	±	2.9 ± 0.3	0.3 ± 0.1
C ₆ H ₅	C ₆ H ₅	CH-OH	±	12.6 ± 0.9	22 ± 2
C ₆ H ₅	C ₆ H ₅	C=NOH	±	3.5 ± 0.5	0.29 ± 0.01
C ₆ H ₅	C ₆ H ₅	S=O	±	2.3 ± 0.4	0.31 ± 0.02
C ₆ H ₅	C ₆ H ₅	CH-OCH ₃	±	103 ± 5	34 ± 1
4-F-C ₆ H ₄	C ₆ H ₅	C=O	±	1.3 ± 0.3	0.3 ± 0.1
4-F-C ₆ H ₄	C ₆ H ₅	CH-OH	±	72 ± 16	18 ± 2
4-C ₆ H ₅ -C ₆ H ₄	C ₆ H ₅	C=O	±	0.14 ± 0.01	0.20 ± 0.01
4-C ₆ H ₅ -C ₆ H ₄	C ₆ H ₅	CH-OH	±	0.51 ± 0.04	0.72 ± 0.03
4-C ₆ H ₅ -C ₆ H ₄	C ₆ H ₅	C=NOH	±	42 ± 3	35 ± 1
4-C ₆ H ₅ -C ₆ H ₄	C ₆ H ₅	S=O	±	73 ± 5	70 ± 3
4-C ₆ H ₅ -C ₆ H ₄	C ₆ H ₅	CH-OCH ₃	±	0.48 ± 0.05	1.36 ± 0.07
C ₁₀ H ₇	C ₆ H ₅	C=O	±	0.49 ± 0.02	0.85 ± 0.06
4-C ₆ H ₅ -C ₆ H ₄	4-CH ₃ -C ₆ H ₄	C=O	±	0.10 ± 0.01	0.19 ± 0.03
4-C ₆ H ₅ -C ₆ H ₄	4-CH ₃ -C ₆ H ₄	CH-OH	±	0.09 ± 0.01	0.15 ± 0.02
4-NO ₂ -C ₆ H ₄	CH ₃	C=O	±	163 ± 11	269 ± 5
4-NO ₂ -C ₆ H ₄	CH ₃	CH-OH	±	6.5 ± 0.2	39 ± 1

C ₆ H ₅	H	CH-OH	<i>R,R</i>	1100 ± 23	
C ₆ H ₅	H	CH-OH	<i>S,S</i>	2400 ± 46	
4-NO ₂ -C ₆ H ₄	H	CH-OH	±	5 ± 1	
4-NO ₂ -C ₆ H ₄	H	CH-OH	<i>R,R</i>	1200 ± 25	
4-NO ₂ -C ₆ H ₄	H	CH-OH	<i>S,S</i>	1.6 ± 0.6	

Abs. Conf.: Absolute configuration

[0011] The enzymes of interest for this invention typically are able to distinguish enantiomers. Thus, in choosing an inhibitor for use for an application in accordance with the invention it is preferred to screen different optical isomers of the inhibitor with the selected enzyme by routine assays so as to choose a better optical isomer, if appropriate, for the particular application. The pharmacophores described here can be used to deliver a reactive functionality to the catalytic site. These could include alkylating agents such as halogens or epoxides or Michael acceptors which will react with thiols and amines. These reactive functionalities also can be used to deliver fluorescent or affinity labels to the enzyme active site for enzyme detection.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figures 1 and 2 show that renal microsomal DHET formation is increased in the SHR relative to the WKY and this is due to increased renal EET hydrolysis. The NADPH-dependent formation of 11,12- DHET (Figure 1A), 8,9-DHET (Figure 1B) and 14,15-DHET (Figure 1C) was measured in incubations of WKY (○) and SHR (◐) renal microsomes with [¹⁴C]-arachidonic acid (Figure 1) or [¹⁴C]-regioisomeric EETs (Figure 2). Values are the mean ± SE from three to six animals of a given age and strain (Figure 1) or means of two separate animals (Figure 2). Significant differences between WKY and SHR samples at a given age are indicated (p<0.05). The hydrolysis of all of the major EETs was increased in the SHR kidney.

[0013] Figure 3 shows that sEH expression is increased in the SHR kidney relative to the WKY kidney and that DHET urinary excretion is also increased in the SHR. (A) Microsomal proteins from WKY (W) and SHR (S) renal cortex were separated on a 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and blotted with antisera against rat mEH (top panel) or mouse sEH (bottom panel). The age of the rats is indicated on the top of the blot. (B) Microsomal (top panel) and cytosolic (bottom panel) proteins from WKY (W) and SHR (S) cortex, outer medulla and liver were separated and transferred as described above

and blotted with antisera against mouse sEH. A renal cortex sample from a Sprague-Dawley (SD) rat and a purified recombinant sEH protein sample are also included on the blot.

Immunoreactive proteins were detected by chemiluminescence. The blots are representative of the results from three to six animals/experimental group. (C) Urine was collected for 24 hr from untreated WKY rats (solid bars) and SHR (hatched bars). DHETs were extracted from urine and quantified by GC-MS as described in the Methods section. The values shown are the means \pm SE of four animals/strain. Significant differences between WKY and SHR are indicated ($p < 0.0005$).

[0014] Figure 4 shows that DCU is a potent and selective inhibitor of sEH and has antihypertensive effects in the SHR. (A) The formation of [1- 14 C]11,12- (A); 8,9- (B), and 14,15-DHET (C) from [1- 14 C]EETs (50 μ M) was measured in SHR renal S9 fractions in the presence of increasing concentrations of DCU. The values shown are the average of two samples/concentration, expressed as % of control. The difference between the individual values was 7-33%. Control formation rates were 7193 pmol/min/mg protein for 14,15-DHET, 538 pmol/min/mg protein for 11,12-DHET, and 595 pmol/min/mg protein for 8,9-DHET. DCU was a potent and selective inhibitor of EET hydrolysis *in vitro*. (B) Urine was collected for 24 hr following treatment of SHR with vehicle (solid bars) or DCU (hatched bars) daily for 3 days. EETs and DHETs were extracted from urine and quantified by GC-MS as described in the Methods section. The values shown are the means \pm SE of four animals/strain. Significant differences between vehicle- and DCU-treated SHR are indicated ($p < 0.05$). DCU was a potent inhibitor of 14,15-EET hydrolysis *in vivo*. (C) Male SHR rats were treated with a single 3 mg/kg dose of DCU (\circ) or vehicle (\bigcirc). Systolic blood pressure was measured with a photoelectric tail cuff for up to 96 hr after the dose. The values shown are the mean \pm SE from DCU- and vehicle-treated rats ($n=5$ /group). Baseline systolic blood pressure was 143 ± 3 mm Hg in the SHRs. (D) Male WKY rats were treated with a single 3 mg/kg dose of DCU (\circ) or vehicle (\bigcirc). Systolic blood pressure was measured with a photoelectric tail cuff for up to 96 hr after the dose. The values shown are the mean \pm SE from DCU- and vehicle-treated rats ($n=5$ /group). Baseline systolic blood pressure was 118 ± 2 mm Hg in the WKY rats. Blood pressure decreased an average of 22 mm Hg in the DCU-treated SHRs 6 hr after the dose ($p < 0.01$) and was unaffected by DCU in the WKY rats.

[0015] Figure 5 shows that a structurally related urea inhibitor of sEH also has antihypertensive effects in the SHR. Male SHRs were treated with a single dose of vehicle (\circ) or N-cyclohexyl-N'-dodecylurea (\circ) (equimolar to 3 mg/kg DCU). Systolic blood,

pressure was measured with a photoelectric tail cuff for 24 hr after the dose. The values shown are the mean \pm SE from inhibitor- and vehicle-treated rats (n=5/group). Baseline systolic blood pressures were 135 ± 5 mm Hg in the N-cyclohexyl-N'-dodecylurea group. Blood pressure decreased an average of 12 mm Hg in the N-cyclohexyl-N'-dodecylurea-treated SHR6s 6 hr after the dose ($p < 0.01$).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0016] The present invention is based on the discovery that epoxide hydrolase activity is associated with hypertension. The epoxyeicosatrienoic acids (EETs) are regarded as antihypertensive eicosanoids due to their potent effects on renal vascular tone and sodium and water transport in the renal tubule. As shown here, EET activity is regulated by hydrolysis to the corresponding dihydroxyeicosatrienoic acids by epoxide hydrolase. Inhibition of EET hydrolysis *in vivo* with a potent and selective soluble epoxide hydrolase inhibitor leads to a decrease in blood pressure. Thus, the present invention provides a new therapeutic approach for the control of blood pressure.

Abbreviations and Definitions:

[0017] The abbreviations used herein have their conventional meaning within the chemical and biological arts.

[0018] The term "alkyl," by itself or as part of another substituent, means, unless otherwise stated, a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof, which may be fully saturated, mono- or polyunsaturated and can include di- and multivalent radicals, having the number of carbon atoms designated (*i.e.* C₁-C₁₀ means one to ten carbons). Examples of saturated hydrocarbon radicals include groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, cyclohexyl, (cyclohexyl)methyl, cyclopropylmethyl, homologs and isomers of, for example, n-pentyl, n-hexyl, n-heptyl, n-octyl, and the like. An unsaturated alkyl group is one having one or more double bonds or triple bonds. Examples of unsaturated alkyl groups include vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butylnyl, and the higher homologs and isomers.

[0019] The term "alkylene" by itself or as part of another substituent means a divalent radical derived from an alkane, as exemplified by -CH₂CH₂CH₂CH₂-, and further includes those groups described below as "heteroalkylene." Typically, an alkyl (or alkylene) group will have from 1 to 24 carbon atoms, with those groups having 10 or fewer carbon atoms

being preferred in the present invention. A "lower alkyl" or "lower alkylene" is a shorter chain alkyl or alkylene group, generally having eight or fewer carbon atoms.

[0020] The terms "cycloalkyl" and "heterocycloalkyl", by themselves or in combination with other terms, represent, unless otherwise stated, cyclic versions of "alkyl" and

5 "heteroalkyl", respectively. Additionally, for heterocycloalkyl, a heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule. Examples of cycloalkyl include cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like. Examples of heterocycloalkyl include 1-(1,2,5,6-tetrahydropyridyl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, 10 tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1-piperazinyl, 2-piperazinyl, and the like.

[0021] The term "aryl" means, unless otherwise stated, a polyunsaturated, typically aromatic, hydrocarbon substituent which can be a single ring or multiple rings (up to three rings) which are fused together or linked covalently. The term "heteroaryl" refers to aryl 15 groups (or rings) that contain from zero to four heteroatoms selected from N, O, and S, wherein the nitrogen and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. A heteroaryl group can be attached to the remainder of the molecule through a heteroatom. Non-limiting examples of aryl and heteroaryl groups include phenyl, 1-naphthyl, 2-naphthyl, 4-biphenyl, 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2- 20 imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxalyl, 5-quinoxalyl, 3-quinolyl, and 6-quinolyl. Substituents for each of the above 25 noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described below.

[0022] For brevity, the term "aryl" when used in combination with other terms (*e.g.*, aryloxy, arylthioxy, arylalkyl) includes both aryl and heteroaryl rings as defined above.

Thus, the term "arylalkyl" is meant to include those radicals in which an aryl group is 30 attached to an alkyl group (*e.g.*, benzyl, phenethyl, pyridylmethyl and the like) including those alkyl groups in which a carbon atom (*e.g.*, a methylene group) has been replaced by, for example, an oxygen atom (*e.g.*, phenoxymethyl, 2-pyridyloxymethyl, 3-(1-naphthyloxy)propyl, and the like).

[0023] Each of the above terms (*e.g.*, “alkyl,” “heteroalkyl,” “aryl” and “heteroaryl”) are meant to include both substituted and unsubstituted forms of the indicated radical. Preferred substituents for each type of radical are provided below.

[0024] Substituents for the alkyl and heteroalkyl radicals (including those groups often referred to as alkylene, alkenyl, heteroalkylene, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, and heterocycloalkenyl) can be a variety of groups selected from: -OR', =O, =NR', =N-OR', -NR'R'', -SR', -halogen, -SiR'R''R''', -OC(O)R', -C(O)R', -CO₂R', -CONR'R'', -OC(O)NR'R'', -NR''C(O)R', -NR'-C(O)NR''R''', -NR''C(O)₂R', -NH-C(NH₂)=NH, -NR'C(NH₂)=NH, -NH-C(NH₂)=NR', -S(O)R', -S(O)₂R', -S(O)₂NR'R'', -CN and -NO₂ in a number ranging from zero to (2m'+1), where m' is the total number of carbon atoms in such radical. R', R'' and R''' each independently refer to hydrogen, unsubstituted (C₁-C₈)alkyl and heteroalkyl, unsubstituted aryl, aryl substituted with 1-3 halogens, unsubstituted alkyl, alkoxy or thioalkoxy groups, or aryl-(C₁-C₄)alkyl groups. When R' and R'' are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6-, or 7-membered ring. For example, -NR'R'' is meant to include 1-pyrrolidinyl and 4-morpholinyl. From the above discussion of substituents, one of skill in the art will understand that the term “alkyl” is meant to include groups such as haloalkyl (*e.g.*, -CF₃ and -CH₂CF₃) and acyl (*e.g.*, -C(O)CH₃, -C(O)CF₃, -C(O)CH₂OCH₃, and the like).

[0025] Similarly, substituents for the aryl and heteroaryl groups are varied and are selected from: -halogen, -OR', -OC(O)R', -NR'R'', -SR', -R', -CN, -NO₂, -CO₂R', -CONR'R'', -C(O)R', -OC(O)NR'R'', -NR''C(O)R', -NR''C(O)₂R', -NR'-C(O)NR''R''', -NH-C(NH₂)=NH, -NR'C(NH₂)=NH, -NH-C(NH₂)=NR', -S(O)R', -S(O)₂R', -S(O)₂NR'R'', -N₃, -CH(Ph)₂, perfluoro(C₁-C₄)alkoxy, and perfluoro(C₁-C₄)alkyl, in a number ranging from zero to the total number of open valences on the aromatic ring system; and where R', R'' and R''' are independently selected from hydrogen, (C₁-C₈)alkyl and heteroalkyl, unsubstituted aryl and heteroaryl, (unsubstituted aryl)-(C₁-C₄)alkyl, and (unsubstituted aryl)oxy-(C₁-C₄)alkyl.

[0026] Two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula -T-C(O)-(CH₂)_q-U-, wherein T and U are independently -NH-, -O-, -CH₂- or a single bond, and q is an integer of from 0 to 2. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula -A-(CH₂)_r-B-, wherein A and B are independently -CH₂-, -O-, -NH-, -S-, -S(O)-, -S(O)₂-, -S(O)₂NR'- or a single bond, and r is an integer of from 1 to 3. One of the single bonds of the new ring so formed may optionally be

replaced with a double bond. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula $-(CH_2)_s-X-(CH_2)_t-$, where s and t are independently integers of from 0 to 3, and X is $-O-$, $-NR'-$, $-S-$, $-S(O)-$, $-S(O)_2-$, or $-S(O)_2NR'-$. The substituent R' in $-NR'-$ and $-S(O)_2NR'-$ is selected from

5 hydrogen or unsubstituted (C_1-C_6) alkyl.

[0027] The terms "halo" or "halogen," by themselves or as part of another substituent, mean, unless otherwise stated, a fluorine, chlorine, bromine, or iodine atom. Additionally, terms such as "haloalkyl," are meant to include monohaloalkyl and polyhaloalkyl. For example, the term "halo (C_1-C_4) alkyl" is meant to include trifluoromethyl, 2,2,2-trifluoroethyl, 10 4-chlorobutyl, 3-bromopropyl, and the like.

[0028] As used herein, the term "heteroatom" is meant to include oxygen (O), nitrogen (N), Boron (B), phosphorous (P) and sulfur (S).

[0029] The term "pharmaceutically acceptable salts" is meant to include salts of the active compounds which are prepared with relatively nontoxic acids or bases, depending on the particular substituents found on the compounds described herein. When compounds of the present invention contain relatively acidic functionalities, base addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired base, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable base addition salts include sodium, potassium, calcium, ammonium, organic amino, or magnesium 15 salt, or a similar salt. When compounds of the present invention contain relatively basic functionalities, acid addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired acid, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable acid addition salts include those derived from inorganic acids like hydrochloric, hydrobromic, nitric, carbonic,

25 monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, hydriodic, or phosphorous acids and the like, as well as the salts derived from relatively nontoxic organic acids like acetic, propionic, isobutyric, maleic, malonic, benzoic, succinic, suberic, fumaric, lactic, mandelic, phthalic, benzenesulfonic, p-tolylsulfonic, citric, tartaric, methanesulfonic, and the like. Also included are salts of amino acids such as arginate and the like, and salts of organic acids like glucuronic or galactunoric acids and the like (see, for example, Berge, S.M., et al, "Pharmaceutical Salts", *Journal of Pharmaceutical Science*, 1977, 66, 1-19). Certain specific compounds of the present invention contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts.

[0030] The neutral forms of the compounds may be regenerated by contacting the salt with a base or acid and isolating the parent compound in the conventional manner. The parent form of the compound differs from the various salt forms in certain physical properties, such as solubility in polar solvents, but otherwise the salts are equivalent to the parent form of the compound for the purposes of the present invention.

[0031] In addition to salt forms, the present invention provides compounds which are in a prodrug form. Prodrugs of the compounds described herein are those compounds that readily undergo chemical changes under physiological conditions to provide the compounds of the present invention. Additionally, prodrugs can be converted to the compounds of the present invention by chemical or biochemical methods in an *ex vivo* environment. For example, prodrugs can be slowly converted to the compounds of the present invention when placed in a transdermal patch reservoir with a suitable enzyme or chemical reagent.

[0032] Certain compounds of the present invention can exist in unsolvated forms as well as solvated forms, including hydrated forms. In general, the solvated forms are equivalent to unsolvated forms and are intended to be encompassed within the scope of the present invention. Certain compounds of the present invention may exist in multiple crystalline or amorphous forms. In general, all physical forms are equivalent for the uses contemplated by the present invention and are intended to be within the scope of the present invention.

[0033] Certain compounds of the present invention possess asymmetric carbon atoms (optical centers) or double bonds; the racemates, diastereomers, geometric isomers and individual isomers are all intended to be encompassed within the scope of the present invention.

Inhibitors of Epoxide Hydrolases

[0034] As noted above, a preferred class of inhibitors of the invention are compounds shown by Formulas 1 and 2, above. Means for preparing such compounds and assaying desired compounds for the ability to inhibit epoxide hydrolases is described in the parent application, USSN 09/252,148. Compounds of Formula 2 are described in 5,955,496 and in WO98/06261.

[0035] In addition to the compounds in Formula 1 which interact with the enzyme in a reversible fashion based on the inhibitor mimicking an enzyme-substrate transition state or reaction intermediate, one can have compounds that are irreversible inhibitors of the enzyme. The active structures such as those in the Tables or Formula 1 can direct the inhibitor to the enzyme where a reactive functionality in the enzyme catalytic site can form a covalent bond

with the inhibitor. One group of molecules which could interact like this would have a leaving group such as a halogen or tosylate which could be attacked in an S_N2 manner with a lysine or histidine. Alternatively, the reactive functionality could be an epoxide or Michael acceptor such as a α/β -unsaturated ester, aldehyde, ketone, ester, or nitrile.

5 [0036] Further, in addition to the Formula 1 compounds, active derivatives can be designed for practicing the invention. For example, dicyclohexyl thio urea can be oxidized to dicyclohexylcarbodiimide which, with enzyme or aqueous acid (physiological saline), will form an active dicyclohexylurea. Alternatively, the acidic protons on carbamates or ureas can be replaced with a variety of substituents which, upon oxidation, hydrolysis or attack by a
10 nucleophile such as glutathione, will yield the corresponding parent structure. These materials are known as prodrugs or protoxins (Gilman et al., *The Pharmacological Basis of Therapeutics*, 7th Edition, MacMillan Publishing Company, New York, p. 16 (1985)) Esters, for example, are common prodrugs which are released to give the corresponding alcohols and acids enzymatically (Yoshigae et al., *Chirality*, 9:661-666 (1997)). The prodrugs can be
15 chiral for greater specificity. These derivatives have been extensively used in medicinal and agricultural chemistry to alter the pharmacological properties of the compounds such as enhancing water solubility, improving formulation chemistry, altering tissue targeting, altering volume of distribution, and altering penetration. They also have been used to alter toxicology profiles.

20 [0037] There are many prodrugs possible, but replacement of one or both of the two active hydrogens in the ureas described here or the single active hydrogen present in carbamates is particularly attractive. Such derivatives have been extensively described by Fukuto and associates. These derivatives have been extensively described and are commonly used in agricultural and medicinal chemistry to alter the pharmacological properties of the
25 compounds. (Black et al., *Journal of Agricultural and Food Chemistry*, 21(5):747-751 (1973); Fahmy et al., *Journal of Agricultural and Food Chemistry*, 26(3):550-556 (1978); Jojima et al., *Journal of Agricultural and Food Chemistry*, 31(3):613-620 (1983); and Fahmy et al., *Journal of Agricultural and Food Chemistry*, 29(3):567-572 (1981).)

[0038] Such active proinhibitor derivatives are within the scope of the present invention,
30 and the just-cited references are incorporated herein by reference. Without being bound by theory, it is believed that suitable inhibitors of the invention mimic the enzyme transition state so that there is a stable interaction with the enzyme catalytic site. The inhibitors appear

to form hydrogen bonds with the nucleophilic carboxylic acid and a polarizing tyrosine of the catalytic site.

[0039] Where the modified activity of the complexed epoxide hydrolase is enzyme inhibition, then particularly preferred compound embodiments have an IC₅₀ (inhibition potency or, by definition, the concentration of inhibitor which reduces enzyme activity by 50%) of less than about 500 μ M.

[0040] Although the preferred inhibitors of the invention specifically inhibit the activity of sEH, some inhibitors of the invention can be used to inhibit the activity of microsomal epoxide hydrolase (mEH). The microsomal enzyme plays a significant role in the metabolism of xenobiotics such as polyaromatic toxicants. Additionally, polymorphism studies have underlined a potential role of this enzyme in relation to several diseases, such as emphysema, spontaneous abortion and several forms of cancer. Inhibition of recombinant rat and human mEH can be obtained using primary ureas, amides, and amines. For example, elaidamide, has a K_i of 70 nM for recombinant rat mEH. This compound interacts with the enzyme forming a non-covalent complex, and blocks substrate turnover through an apparent mix of competitive and non-competitive inhibition kinetics. Furthermore, in insect cell culture expressing rat mEH, elaidamide enhances the toxicity effects of epoxide-containing xenobiotics.

Assays for Epoxide Hydrolase Activity

[0041] The invention also provides methods for assaying for epoxide hydrolase activity as diagnostic assay to identify individuals at increased risk for hypertension and/or those that would benefit from the therapeutic methods of the invention. Any of a number of standard assays for determining epoxide hydrolase activity can be used. For example, suitable assays are described in Gill, *et al.*, *Anal Biochem* 131, 273-282 (1983); and Borhan, *et al.*, *Analytical Biochemistry* 231, 188-200 (1995)). Suitable *in vitro* assays are described in Zeldin *et al.* *J Biol. Chem.* 268:6402-6407 (1993). Suitable *in vivo* assays are described in Zeldin *et al.* *Arch Biochem Biophys* 330:87-96 (1996). Assays for epoxide hydrolase using both putative natural substrates and surrogate substrates have been reviewed (*see*, Hammock, *et al.* *In: Methods in Enzymology, Volume III, Steroids and Isoprenoids, Part B*, (Law, J.H. and H.C. Rilling, eds. 1985), Academic Press, Orlando, Florida, pp. 303-311 and Wixtrom *et al.*, *In: Biochemical Pharmacology and Toxicology, Vol. 1: Methodological Aspects of Drug Metabolizing Enzymes*, (Zakim, D. and D.A. Vessey, eds. 1985), John Wiley & Sons, Inc.,

New York, pp. 1-93. Several spectral based assays exist based on the reactivity or tendency of the resulting diol product to hydrogen bond (*see, e.g.,* Wixtrom, and Hammock. *Anal. Biochem.* 174:291-299 (1985) and Dietze, *et al. Anal. Biochem.* 216:176-187 (1994)).

[0042] The enzyme also can be detected based on the binding of specific ligands to the catalytic site which either immobilize the enzyme or label it with a probe such as luciferase, green fluorescent protein or other reagent. For the data in this disclosure the enzyme was assayed by its hydration of EETs, its hydrolysis of an epoxide to give a colored product as described by Dietze et al. (1994) or its hydrolysis of a radioactive surrogate substrate (Borhan et al., 1995)

[0043] The assays of the invention are carried out using an appropriate sample from the patient. Typically, such a sample is a blood sample.

Other Means of inhibiting EH activity

[0044] Other means of inhibiting EH activity or gene expression can also be used. For example, a nucleic acid molecule complementary to at least a portion of the human EH gene can be used to inhibit EH gene expression. Means for inhibiting gene expression using, for example, antisense molecules, ribozymes, and the like are well known to those of skill in the art. The nucleic acid molecule can be a DNA probe, a riboprobe, a peptide nucleic acid probe, a phosphorothioate probe, or a 2'-O methyl probe.

[0045] Generally, to assure specific hybridization, the antisense sequence is substantially complementary to the target sequence. In certain embodiments, the antisense sequence is exactly complementary to the target sequence. The antisense polynucleotides may also include, however, nucleotide substitutions, additions, deletions, transitions, transpositions, or modifications, or other nucleic acid sequences or non-nucleic acid moieties so long as specific binding to the relevant target sequence corresponding to the EH gene is retained as a functional property of the polynucleotide. As one embodiment of the antisense molecules form a triple helix-containing, or "triplex" nucleic acid. Triple helix formation results in inhibition of gene expression by, for example, preventing transcription of the target gene (*see, e.g.,* Cheng *et al.*, 1988, *J. Biol. Chem.* 263:15110; Ferrin and Camerini-Otero, 1991, *Science* 254:1494; Ramdas *et al.*, 1989, *J. Biol. Chem.* 264:17395; Strobel *et al.*, 1991, *Science* 254:1639; and Rigas *et al.*, 1986, *Proc. Natl. Acad. Sci. U.S.A.* 83:9591)

[0046] In another embodiment, ribozymes can be used (*see, e.g.,* Cech, 1995, *Biotechnology* 13:323; and Edgington, 1992, *Biotechnology* 10:256 and Hu *et al.*, PCT Publication WO 94/03596).

[0047] The antisense nucleic acids (DNA, RNA, modified, analogues, and the like) can be made using any suitable method for producing a nucleic acid, such as the chemical synthesis and recombinant methods disclosed herein and known to one of skill in the art. In one embodiment, for example, antisense RNA molecules of the invention may be prepared by *de novo* chemical synthesis or by cloning. For example, an antisense RNA can be made by inserting (ligating) an EH gene sequence in reverse orientation operably linked to a promoter in a vector (*e.g.*, plasmid). Provided that the promoter and, preferably termination and polyadenylation signals, are properly positioned, the strand of the inserted sequence corresponding to the noncoding strand will be transcribed and act as an antisense oligonucleotide of the invention.

[0048] It will be appreciated that the oligonucleotides can be made using nonstandard bases (*e.g.*, other than adenine, cytidine, guanine, thymine, and uridine) or nonstandard backbone structures to provides desirable properties (*e.g.*, increased nuclease-resistance, tighter-binding, stability or a desired T_m). Techniques for rendering oligonucleotides nuclease-resistant include those described in PCT Publication WO 94/12633. A wide variety of useful modified oligonucleotides may be produced, including oligonucleotides having a peptide-nucleic acid (PNA) backbone (Nielsen *et al.*, 1991, *Science* 254:1497) or incorporating 2'-O-methyl ribonucleotides, phosphorothioate nucleotides, methyl phosphonate nucleotides, phosphotriester nucleotides, phosphorothioate nucleotides, phosphoramidates.

[0049] Proteins have been described that have the ability to translocate desired nucleic acids across a cell membrane. Typically, such proteins have amphiphilic or hydrophobic subsequences that have the ability to act as membrane-translocating carriers. For example, homeodomain proteins have the ability to translocate across cell membranes. The shortest internalizable peptide of a homeodomain protein, Antennapedia, was found to be the third helix of the protein, from amino acid position 43 to 58 (*see, e.g.*, Prochiantz, 1996, *Current Opinion in Neurobiology* 6:629-634. Another subsequence, the h (hydrophobic) domain of signal peptides, was found to have similar cell membrane translocation characteristics (*see, e.g.*, Lin *et al.*, 1995, *J. Biol. Chem.* 270:14255-14258). Such subsequences can be used to translocate oligonucleotides across a cell membrane. Oligonucleotides can be conveniently derivatized with such sequences. For example, a linker can be used to link the oligonucleotides and the translocation sequence. Any suitable linker can be used, *e.g.*, a peptide linker or any other suitable chemical linker.

Therapeutic Administration

[0050] The compounds of the present invention can be prepared and administered in a wide variety of oral, parenteral and topical dosage forms. Thus, the compounds of the present invention can be administered by injection, that is, intravenously, intramuscularly, intracutaneously, subcutaneously, intraduodenally, or intraperitoneally. Also, the compounds described herein can be administered by inhalation, for example, intranasally. Additionally, the compounds of the present invention can be administered transdermally. Accordingly, the present invention also provides pharmaceutical compositions comprising a pharmaceutically acceptable carrier or excipient and either a compound of the invention or a pharmaceutically acceptable salt of the compound.

[0051] For preparing pharmaceutical compositions from the compounds of the present invention, pharmaceutically acceptable carriers can be either solid or liquid. Solid form preparations include powders, tablets, pills, capsules, cachets, suppositories, and dispersible granules. A solid carrier can be one or more substances which may also act as diluents, flavoring agents, binders, preservatives, tablet disintegrating agents, or an encapsulating material.

[0052] In powders, the carrier is a finely divided solid which is in a mixture with the finely divided active component. In tablets, the active component is mixed with the carrier having the necessary binding properties in suitable proportions and compacted in the shape and size desired.

[0053] The powders and tablets preferably contain from 5% or 10% to 70% of the active compound. Suitable carriers are magnesium carbonate, magnesium stearate, talc, sugar, lactose, pectin, dextrin, starch, gelatin, tragacanth, methylcellulose, sodium carboxymethylcellulose, a low melting wax, cocoa butter, and the like. The term "preparation" is intended to include the formulation of the active compound with encapsulating material as a carrier providing a capsule in which the active component with or without other carriers, is surrounded by a carrier, which is thus in association with it. Similarly, cachets and lozenges are included. Tablets, powders, capsules, pills, cachets, and lozenges can be used as solid dosage forms suitable for oral administration.

[0054] For preparing suppositories, a low melting wax, such as a mixture of fatty acid glycerides or cocoa butter, is first melted and the active component is dispersed homogeneously therein, as by stirring. The molten homogeneous mixture is then poured into convenient sized molds, allowed to cool, and thereby to solidify.

[0055] Liquid form preparations include solutions, suspensions, and emulsions, for example, water or water/propylene glycol solutions. For parenteral injection, liquid preparations can be formulated in solution in aqueous polyethylene glycol solution.

[0056] Aqueous solutions suitable for oral use can be prepared by dissolving the active component in water and adding suitable colorants, flavors, stabilizers, and thickening agents as desired. Aqueous suspensions suitable for oral use can be made by dispersing the finely divided active component in water with viscous material, such as natural or synthetic gums, resins, methylcellulose, sodium carboxymethylcellulose, and other well-known suspending agents.

[0057] Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations for oral administration. Such liquid forms include solutions, suspensions, and emulsions. These preparations may contain, in addition to the active component, colorants, flavors, stabilizers, buffers, artificial and natural sweeteners, dispersants, thickeners, solubilizing agents, and the like.

[0058] The pharmaceutical preparation is preferably in unit dosage form. In such form the preparation is subdivided into unit doses containing appropriate quantities of the active component. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, such as packeted tablets, capsules, and powders in vials or ampoules. Also, the unit dosage form can be a capsule, tablet, cachet, or lozenge itself; or it can be the appropriate number of any of these in packaged form.

[0059] The term "unit dosage form", as used in the specification, refers to physically discrete units suitable as unitary dosages for human subjects and animals, each unit containing a predetermined quantity of active material calculated to produce the desired pharmaceutical effect in association with the required pharmaceutical diluent, carrier or vehicle. The specifications for the novel unit dosage forms of this invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular effect to be achieved and (b) the limitations inherent in the art of compounding such an active material for use in humans and animals, as disclosed in detail in this specification, these being features of the present invention.

[0060] A therapeutically effective amount of the compounds of the invention is employed in treatment. The dosage of the specific compound for treatment depends on many factors that are well known to those skilled in the art. They include for example, the route of administration and the potency of the particular compound. An exemplary dose is from about

0.001 $\mu\text{M/kg}$ to about 100 mg/kg body weight of the mammal. Without further elaboration, it is believed that one skilled in the art can, using the preceding description, practice the present invention to its fullest extent. The following detailed examples describe how to prepare the various compounds and/or perform the various processes of the invention and are to be construed as merely illustrative, and not limitations of the preceding disclosure in any way whatsoever. Those skilled in the art will promptly recognize appropriate variations from the procedures both as to reactants and as to reaction conditions and techniques.

EXAMPLE

[0061] This example shows that inhibitors of epoxide hydrolase are effective in decreasing blood pressure in mammals.

METHODS

[0062] Animals. Male SHR and WKY rats 3-13 wks of age were purchased from Charles River Laboratories (Wilmington, MA) and housed in a controlled environment with 12 hr light/dark cycles and fed standard laboratory chow for \square 3 days before euthanasia. All animal use was approved by the University of California San Francisco Committee on Animal Research and followed the National Institutes of Health guidelines for the care and use of laboratory animals. For isolation of kidney subcellular fractions, rats were anesthetized with methoxyflurane, the abdominal cavities were opened, and the kidneys were perfused with ice-cold saline. Perfused kidneys were rapidly removed, the cortex and medulla dissected out and immersed in liquid nitrogen. All tissue was stored at -80°C until preparation of microsomes. In some cases WKY and SHR rats were housed in metabolic cages for up to three days and urine was collected over triphenylphosphine in 24 hr intervals. The urine volume was noted and aliquots were stored at -80°C prior to extraction and quantitation of DHETs and EETs. For the sEH inhibition studies, groups of 8 wk old male SHRs and WKY rats were treated daily for 1-4 days with a 3 mg/kg i.p. dose of N,N'-dicyclohexylurea (DCU) in a 1.5:1 mixture of corn oil and DMSO. Systolic blood pressure was measured at room temperature by a photoelectric tail cuff system (Model 179, IITC, Inc., Woodland Hills, CA) for up to four days following the dose of inhibitor. Blood pressures are reported as the average of three separate readings over a 30 min period. Urine was collected for 24 hr immediately following a dose of DCU or vehicle for quantification of DHET and

EET excretion. Similar inhibition studies were carried out with equimolar doses of N-cyclohexyl-N'-dodecylurea, N-cyclohexyl-N'-ethylurea and dodecylamine.

[0063] Renal microsomal arachidonic acid metabolism. Microsomes, cytosol, and S9 fractions were prepared from the renal cortex or outer medulla samples from a single animal as described previously (Kroetz, D.L. et al. *Mol Pharmacol* 52, 362-372 (1997), Su, P. et al. *Am J Physiol* 275, R426-438 (1998)). Reaction conditions for the in vitro determination of arachidonic acid epoxygenase activity, metabolite extraction and HPLC analysis were described in detail elsewhere (Su, P. et al. *Am J Physiol* 275, R426-438 (1998)).

[0064] Western immunoblotting. Renal and hepatic microsomes and cytosol (4 to 10 μ g) were separated on a 8% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose in 25 mM Tris/192 mM glycine/20% methanol using a semidry transfer system (BioRad, Hercules, CA). The primary antibody used in these studies was a rabbit anti-mouse sEH antisera (Silva, M.H. et al. *Comp. Biochem Physiol B: Comp Biochem* 87, 95-102 (1987)). Western blots were incubated with a 1:2000 fold dilution of the primary antibody followed by a 1:2000-fold dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG. Immunoreactive proteins were visualized using an ECL detection kit (Amersham Life Science, Arlington Heights, IL).

[0065] EET hydrolysis. Racemic [1-14C]EETs were synthesized and purified according to published methods from [1-14C]arachidonic acid (56-57 μ Ci/ μ mole) by nonselective epoxidation (Falck, J.R. et al., *Meth Enzymol* 187, 357-364 (1990)). Hydrolysis of [1-14C]EETs was measured in WKY and SHR renal S9 fractions at 37°C as described previously (Zeldin, D.C. et al. *J Biol Chem* 268, 6402-6407 (1993)). The reaction mixture consisted of 50 μ M EET (0.045-0.09 μ Ci) and 1 mg/ml S9 protein (0.5 mg/ml SHR S9 protein for 14,15-EET hydrolysis) in 150 mM KCl, 10 mM MgCl₂, 50 mM potassium phosphate buffer pH 7.4. Reactions were carried out for 40 min (10 min for 14,15-EET hydrolysis in SHR samples) and the reaction products were extracted into ethyl acetate, evaporated under a blanket of nitrogen and detected by reverse phase HPLC with radiometric detection as described for the arachidonic acid incubations.

[0066] DHET urinary excretion. Urinary creatinine concentrations were measured by the Medical Center Clinical Laboratories at the University of California San Francisco. Methods used to quantify endogenous EETs and DHETs present in rat urine were similar to those described by Capdevila et al. (Capdevila, J.H. et al. *J Biol Chem* 267, 21720-21726 (1992)). DHET and [1-14C]DHET internal standards were prepared by chemical hydration of EETs and [1-14C]EETs as described (Zeldin, D.C. et al. *J Biol Chem* 268, 6402-6407 (1993)). All

synthetic EETs and DHETs were purified by reverse-phase HPLC. EET quantifications were made by GC/MS analysis of their pentafluorobenzyl (PFB) esters with selected ion monitoring at m/z 319 (loss of PFB from endogenous EET-PFB) and m/z 321 (loss of PFB from [1- ^{14}C]EET-PFB internal standard). The EET-PFB/[1- ^{14}C]EET-PFB ratios were

calculated from the integrated values of the corresponding ion current intensities.

Quantifications of DHETs were made from GC/MS analysis of their PFB esters, trimethylsilyl (TMS) ethers with selected ion monitoring at m/z 481 (loss of PFB from endogenous DHET-PFB-TMS) and m/z 483 (loss of PFB from [1- ^{14}C]DHET-PFB-TMS internal standard). The DHET-PFB-TMS/[1- ^{14}C]DHET-PFB-TMS ratios were calculated from the integrated values of the corresponding ion current intensities. Data were normalized for kidney function by expressing per mg creatinine. Control studies demonstrated that under the conditions used, artifactual EET or DHET formation was negligible.

[0067] Other enzyme assays. Activities of microsomal and soluble EH were determined in liver and kidney samples according to previously published protocols (Gill, S.S. et al., *Anal Biochem* 131, 273-282 (1983); Borhan, B., et al., *Anal Biochem* 231, 188-200 (1995)). Inhibition of recombinant soluble EH by DCU was described recently (Morisseau, C., et al. *Proc. Natl. Acad. Sci. USA* 96, 8849-8854, (1999)). Epoxide hydrolase activities are reported as the transdiol formation rates.

[0068] Statistics. Statistical significance of differences between mean values was evaluated by a one-way analysis of variance or a Student's t-test. Significance was set at a p value of < 0.05 .

RESULTS

[0069] The spontaneously hypertensive rat (SHR) is a well accepted experimental model of essential hypertension and was used in the present study to characterize the contribution of EET hydrolysis to the elevated blood pressure in these animals. Renal transplantation studies support a role for the kidneys in the development of hypertension in the SHR and altered renal function is essential for the development and maintenance of elevated blood pressure (Bianchi, G. et al., *Clin Sci Mol Med* 47, 435-448 (1974); Cowley, A.W. et al., *JAMA* 275, 1581-1589 (1996)). Arachidonic acid metabolism was measured in renal cortical microsomes of SHR and WKY rats and a dramatic increase in DHET formation was observed in the SHR relative to the WKY samples (Figure 1). The formation of 11,12- and 8,9-DHET was measurable in both strains and was 2- to 8-fold higher in the SHR relative to the WKY rat throughout their development (Figures 1A and 1B). Interestingly, 14,15-DHET formation

was readily detected in the 3-13 wk old SHR kidneys but could not be measured in the majority of the WKY samples (Figure 1C). In the several instances where 14,15-DHET formation was detectable in the WKY kidneys it was never greater than 17% of the corresponding value in SHR. Calculation of the percentage of EETs that were converted to the corresponding DHETs revealed a large discrepancy between the WKY and SHR strains. In the WKY renal microsomes $32 \pm 3.1\%$ of the EETs were converted to DHETs, while the DHET recovery was $66 \pm 2.1\%$ in the SHR renal microsomes ($p < 0.00001$).

[0070] A dramatic increase in DHET formation in incubations of arachidonic acid with SHR renal cortical microsomes relative to the WKY samples (Figure 1) led us to hypothesize that EET hydrolysis may be altered in this experimental model of hypertension and that epoxide hydrolase activity may be an important determinant of blood pressure regulation. Two major EH isoforms, a microsomal (mEH) and soluble (sEH) form are expressed in most tissues and species (Vogel-Bindel, U. et al., *Eur J Biochem* 126, 425-431 (1982); Kaur, S. et al., *Drug Metab Disp* 13, 711-715 (1985)). EET hydrolysis rates in cytosol and microsomes and the regioisomeric product distribution in urine relative to recombinant EH proteins are consistent with the majority of EET hydrolysis being catalyzed by sEH (Zeldin, D.C. et al., *J Biol Chem* 268, 6402-6407, (1993)). Direct hydrolysis of the regioisomeric EETs was measured in S9 fractions (containing both the soluble and microsomal forms of EH) from WKY and SHR renal cortex. There was measurable hydrolysis of 8,9-, 11,12- and 14,15-EET in S9 fractions from both WKY and SHR kidneys (Figure 2), with a significant increase in hydrolysis in the SHR relative to the WKY. For example, 8,9- and 11,12-EET hydrolysis rates were 5- to 15-fold higher in the SHR compared to the WKY and 14,15-EET hydrolysis was as much as 54-fold higher in the SHR. These data also showed a distinct preference of sEH for the 14,15-EET regioisomer. In the SHR kidney hydrolysis of 14,15-EET was 10-fold higher than that of 8,9- and 11,12-EET.

[0071] To investigate the possibility that altered EH expression is responsible for the differences in EET hydrolysis in the SHR and WKY rat renal microsomes and S9 fractions, we measured EH protein levels in these samples. mEH was abundantly expressed in the renal microsomes at relatively constant levels throughout development and there was no evidence of altered expression of mEH in the SHR kidney (Figure 3A). The soluble EH isoform was also easily detected in SHR cortical microsomes but not in the corresponding WKY samples (Figure 3A). Quantitation of the immunoreactive protein bands indicated that levels of sEH protein in the SHR microsomes were 6- to 90-fold higher than the corresponding levels in the WKY microsomes. The high levels of expression of sEH in the SHR microsomes were

limited to the renal cortex (Figure 3B). Immunodetectable sEH was barely detectable in SHR outer medulla and liver microsomes by Western blot. Relatively high levels of sEH were detected in SHR cortex, outer medulla and liver cytosol (Figure 3B). In the WKY rats, the level of sEH protein was uniformly low in both microsomes and cytosol from the kidney and liver. Importantly, sEH protein in the normotensive Sprague-Dawley rat kidney was also barely detectable. Increased sEH expression in SHR vs. WKY rats provides an explanation for the increased EET hydrolysis in the SHR kidney and the absence or very low levels of 14,15-EET hydrolysis, the preferred sEH substrate (Zeldin, D.C., et al., J Biol Chem 268, 6402-6407 (1993)), in the WKY kidney.

[0072] Increased sEH activity in the SHR kidney was independently confirmed using the sEH substrate trans-1,3-diphenylpropene oxide (tDPPO). There was a 26-fold increase in tDPPO hydrolysis in the SHR cortical cytosol relative to that of the WKY rat cortical cytosol (Table 2). The corresponding difference in the microsomal fraction was 32-fold. Hydrolysis of tDPPO was also significantly higher in SHR vs. WKY rat liver microsomes and cytosol.

Consistent with the Western blots, sEH activity was easily detectable in the SHR microsomes and very low in the WKY cytosol and microsomes from kidney. In contrast, mEH activity, as measured by cSO hydrolysis, was similar in WKY and SHR cortex and liver (Table 2).

[0073] Urinary excretion of DHETs was measured to evaluate whether increased sEH expression and EET hydrolysis in the SHR was also apparent in vivo. Urine was collected in untreated 4 and 8 wk old SHR and WKY rats and their DHET excretion rates are shown in Figure 3C. The excretion rates were similar for the 4 and 8 wk animals and the reported numbers are averages from all samples of a given strain. The excretion of 14,15-DHET was 2.6-fold higher in the SHR relative to the WKY rat, consistent with the increased EET hydrolysis and sEH expression in SHR kidney. In contrast, the 8,9- and 11,12-DHET urinary excretion in the SHR and WKY rats were comparable.

[0074] A tight binding sEH specific inhibitor, dicyclohexylurea (DCU) (Morisseau, C. et al., Proc Natl Acad Sci USA 96, 8849-8854 (1999)), was used to reduce sEH activity in vivo and to determine the effect of decreased EET hydrolysis on blood pressure. Inhibition of EET hydrolysis by DCU was confirmed in incubations of renal S9 fractions with the regioisomeric EETs (Figure 4A). A dose-dependent inhibition of EET hydrolysis by DCU was apparent for all three regioisomers. DCU had the most significant effect on the hydrolysis of 8,9-EET, inhibiting this reaction with an IC_{50} of $0.086 \pm 0.014 \mu M$. The corresponding IC_{50} values for inhibition of 11,12- and 14,15-EET hydrolysis were $0.54 \pm 0.08 \mu M$ and $0.45 \pm 0.16 \mu M$, respectively. At concentrations up to $25 \mu M$, DCU had no

effect on CYP epoxigenase or ω -hydroxylase activity and previous studies from our laboratory have shown that DCU does not inhibit mEH (Morisseau, C. et al., Proc Natl Acad Sci USA 96, 8849-8854 (1999)). The potent inhibition of sEH by DCU was confirmed with purified recombinant rat sEH. DCU inhibited sEH-catalyzed tDPPO hydrolysis with a K_i of 34 nM. This is comparable to the K_i values for DCU with human (30 nM) and murine (26 nM) sEH (Morisseau, C. et al., Proc Natl Acad Sci USA 96, 8849-8854 (1999)).

[0075] DCU was administered to eight wk old SHR daily for four days and urinary DHET excretion was measured during the 24 hr period immediately following the third dose. The dose of DCU was based on in vitro estimates of inhibitory potency and previous studies in the mouse (Morisseau, C. et al., Proc Natl Acad Sci USA 96, 8849-8854 (1999)). In the DCU-treated rats there was a significant 65% decrease in 14,15-DHET urinary excretion and a corresponding 30% increase in 14,15-EET urinary excretion relative to vehicle-treated controls (Figure 4B), consistent with DCU-mediated inhibition of sEH in vivo. The excretion of total epoxigenase-derived products (EETs and DHETs) was decreased from 2020 pg/mg creatinine in the vehicle-treated animals to 1237 pg/mg creatinine in the DCU-treated rats ($p < 0.05$). This inhibition of 14,15-DHET excretion was accompanied by a significant decrease in blood pressure measured in conscious animals three to five hr after the fourth dose. Systolic blood pressure decreased from 128 ± 5 mm Hg in the vehicle-treated rats to 102 ± 5 mm Hg ($p < 0.01$) in the DCU-treated animals.

[0076] A study of the time course of the effect of a single dose of DCU (3 mg/kg) demonstrated that the antihypertensive effect in the SHR was acute (Figure 4C). Blood pressure was decreased 22 ± 4 mm Hg 6 hr after DCU treatment ($p < 0.01$) and returned to baseline levels by 24 hr after the dose. Importantly, there was no effect of DCU on blood pressure in the WKY (Figure 4D). This is consistent with the very low levels of sEH protein in the WKY kidney. Several additional structurally related inhibitors were also studied in the SHR. N-cyclohexyl-N'-dodecylurea is a sEH inhibitor with similar potency to DCU (IC_{50} with mouse sEH = 0.05 ± 0.01 compared to 0.09 ± 0.01 μ M for DCU; unpublished data, C. Morisseau and B. Hammock, 2000). A single dose of N-cyclohexyl-N'-dodecylurea significantly decreased systolic blood pressure 12 ± 2 mm Hg 6 hr after the dose, and similar to DCU, blood pressure returned to normal by 24 hours after the dose (Figure 5). The N-cyclohexyl-N'-ethylurea analog is a weak sEH inhibitor (IC_{50} with mouse sEH = 51.7 ± 0.7 μ M; unpublished data, C. Morisseau and B. Hammock, 2000) and had no effect on blood pressure in the SHR. Likewise, the selective mEH inhibitor dodecylamine also had no effect on blood pressure. Collectively, these data suggest that the effect of DCU and N-cyclohexyl-

N'-dodecylurea on blood pressure is related to their ability to inhibit sEH and EET hydrolysis in vivo.

DISCUSSION

[0077] The EET eicosanoids are recognized as important mediators of vascular tone and renal tubular sodium and water transport (Makita, K. et al., *FASEB J* **10**, 1456-1463 (1996)). These data provide substantial evidence in support of the protective role of the EETs. The potential protective effects of increased EET formation in the SHR kidney are attenuated by an even greater increase in FET hydrolysis. Increased expression of sEH in the SHR kidney results in increased EET hydrolysis *in vitro* and *in vivo* and therefore lower levels of the antihypertensive EETs. In contrast, inhibition of EET hydrolysis *in vivo* is associated with elevated EET levels and a reduction in blood pressure. Importantly, this provides a common link between the pathophysiological regulation of blood pressure in rats and humans (Catella, F. et al., *Proc Natl Acad Sci USA* **87**, 5893-5897 (1990)). The 8,9-DHET regioisomer is easily detected in the urine of healthy women while excretion of 14,15- and 11,12-DHET is minimal in this population. For all three isomers DHET excretion is increased during a normal pregnancy and during pregnancy-induced hypertension 14,15- and 11,12-DHET excretion increased even further. This was most dramatic for 14,15-EET, the preferred substrate for sEH (Zeldin, D.G. et al., *J Biol Chem* **268**, 6402-6407 (1993)). The excretion of 14,15-DHET increased from a median of 85 pg/mg of creatinine during normal pregnancy to 2781 pg/mg of creatinine in women with pregnancy-induced hypertension. Pharmacokinetic evidence is consistent with a renal origin of the urinary DHETs (Catella, F. et al., *Proc Natl Acad Sci USA* **87**, 5893-5897 (1990)) so altered EET and DHET levels could potentially affect tubular ion transport and/or renal vascular tone. The present results in the SHR support the possibility that sEH expression is altered in women with pregnancy induced hypertension.

[0078] Inhibition of EET hydrolysis is a new therapeutic approach to regulating renal eicosanoid formation. Recently, inhibition of arachidonic acid ω -hydroxylase activity with a mechanism-based CYP inhibitor has been shown to effectively lower blood pressure in the SHR (Su, P. et al., *Am J Physiol* **275**, R426-438 (1998)). The approach of inhibition of EET hydrolysis in the present study produces a significantly greater decrease in blood pressure than the CYP inhibition strategy. The possibility exists of a synergistic effect of CYP4A inhibition resulting in decreased levels of the prohypertensive 20-HETE eicosanoid and sEH inhibition leading to increased levels of the antihypertensive EETs. Parallel inhibition of related enzymes is a limitation of CYP epoxygenase and ω -hydroxylase inhibition but is of

little concern with sEH inhibition. These findings make it of interest to fully characterize the impact of sEH inhibition on renal tubular ion transport, vascular tone and blood pressure.

The possibility of similar changes in sEH activity in human hypertensive populations is compelling. Identification of individuals with elevated sEH activity may prove useful in

5 designing the most effective antihypertensive therapy.

[0079] It is to be understood that while the invention has been described above in conjunction with preferred specific embodiments, the description and examples are intended to illustrate and not limit the scope of the invention, which is defined by the scope of the
10 appended claims. All publications, sequences referred to in GenBank accession numbers, patents, and patent applications cited herein are hereby incorporated by reference for all purposes.